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Comparison of Effects of Medium Composition and Atmospheric Conditions on Detection of *Bilophila wadsworthia* β-Lactamase by Cefinase and Cefinase Plus Methods

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The influence of growth medium and incubation conditions on the detection of *Bilophila wadsworthia* β-lactamase was tested with Cefinase and Cefinase Plus disks. The tests involved aerobic and anaerobic incubation with conventional disk and quantitative tube assays. The production of β-lactamase was correlated with penicillin G, ampicillin, and ampicillin-sulbactam MICs and inhibition zones on penicillin (2-U) disks. The strains were grown on (i) brucella agar (brucella), (ii) brucella agar supplemented with 1% pyruvate (brucella-pyruvate), and (iii) brucella agar supplemented with 1% taurine (brucella-taurine). With the aerobic disk assay, 100, 100, and 7% of strains were positive after 30 min from growth on brucella-pyruvate, brucella, and brucella-taurine plates, respectively; of strains grown on brucella-taurine, 54% remained negative by the Cefinase assay, and 23% remained negative by the Cefinase Plus assay at 2 h. In quantitative assays, the strains became positive after 30 min from brucella-pyruvate plates and after 1 h from brucella plates. The intensities of the reactions were strongest with brucella-pyruvate plates under anaerobic test conditions. Anaerobic incubation enhanced β-lactamase detection of growth on brucella-taurine: at 3 h, 85% of strains were positive in comparison to 38% with aerobic incubation. All β-lactamase-negative strains were susceptible to penicillin G and ampicillin; all β-lactamase-positive strains were resistant to ampicillin and, with the exception of two strains, penicillin G. In conclusion, β-lactamase production correlated with susceptibility to penicillin G and ampicillin. Brucella agar supplemented with 1% pyruvate was the most reliable medium for testing *B. wadsworthia* β-lactamase, and anaerobic incubation expedited positive results. Brucella agar supplemented with taurine was unsuitable for *B. wadsworthia* β-lactamase testing. Cefinase and Cefinase Plus results were in agreement, but Cefinase Plus yielded faster reactions.

Bilophila wadsworthia is an anaerobic, gram-negative rod originally isolated from patients with appendicitis and related clinical conditions (1, 2). Since those reports, its isolation from a wide variety of clinical infections, including bacteremia and brain and liver abscesses, has been described (5, 14). β-Lactamase production occurs with high frequency in *B. wadsworthia* (12, 13), but the demonstration of *B. wadsworthia* β-lactamase has been problematic and inconsistent. Initially, *B. wadsworthia* was reported as negative for β-lactamase production (1), and one report failed to demonstrate β-lactamase production by *B. wadsworthia* although the antibiogram obtained suggested resistance due to β-lactamase (11). However, β-lactamase production was demonstrated in the presence of pyruvate (13), and the enzyme was characterized to be a cephalosporinase (P. Summanen, H. M. Wexler, and S. M. Finegold, unpublished data). Growth conditions as well as aerobic testing conditions have been found to be possible influences on the detection of *B. wadsworthia* β-lactamase (13; Summanen et al., unpublished data).

The present study was undertaken to find a method that reliably allows the demonstration of *B. wadsworthia* β-lactamase activity in a clinical laboratory setting. Cefinase and Cefinase Plus disks were used for β-lactamase detection. The influence of growth medium was tested on (i) ready-made commercially available brucella agar; (ii) brucella agar supplemented with 1% pyruvate, which is the recommended medium for *B. wadsworthia* susceptibility testing (13); and (iii) brucella agar supplemented with 1% taurine (an amino acid found as a constituent of bile), which provides luxuriant growth of *B. wadsworthia* (H. Laue, U. K. Schumacher, and A. M. Cook, Abstr. Anaerobe Soc. Am. Congr. Anaerobic Bacteria 1998, p. 11). The tests were performed under aerobic and anaerobic conditions. β-Lactamase production was correlated with susceptibility to penicillin G, ampicillin, and ampicillin-sulbactam.

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MATERIALS AND METHODS

Bacterial strains. Thirty-eight β-lactamase-positive and three β-lactamase-negative clinical strains of *B. wadsworthia* isolated from various clinical specimens, including appendix tissue (including ATCC 49260 [WAL 7959]), peritoneal fluid, intra-abdominal abscess, necrotizing fasciitis, head wound, joint fluid, axillary wound, and knee drainage fluid, were tested. Organisms were maintained at −70°C in double-strength skim milk. For β-lactamase testing, the strains were subcultured twice on (i) brucella agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% laked sheep blood, 5 μg of hemin per ml, 1 μg of vitamin K_1 per ml, and 1% pyruvic acid (Sigma Chemical Co., St. Louis, Mo.) (brucella-pyruvate); (ii) brucella agar (BBL) supplemented with 5% laked sheep blood, 5 μg of hemin per ml, 1 μg of vitamin K_1 per ml, and 1% taurine (Sigma) (brucella-taurine); and (iii) ready-made commercially available brucella agar plates supplemented with 5% sheep blood, 5 μg of hemin per ml, and 1 μg of vitamin K_1 per ml (BBL) (brucella). All tests were performed with subcultures from the respective brucella blood agar.

Chemicals. Cefinase, Cefinase Plus, and penicillin (2-U) disks were obtained from BBL Microbiology Systems, and triphenyltetrazolium chloride was obtained from Sigma Chemical Co. Ampicillin (Pfizer Inc., New York, N.Y.), penicillin G (Eli Lilly and Company, Indianapolis, Ind.), and sulbactam (Pfizer) were obtained from the manufacturers and prepared by the methods outlined in the National Committee for Clinical Laboratory Standards (NCCLS) reference method (8).

Cefinase and Cefinase Plus quantitative assays. Fourteen strains were grown for 48 h on brucella, brucella-pyruvate, and brucella-taurine media. A heavy suspension (McFarland standard of 5 to 6) of organisms on each medium was prepared in distilled water. Two hundred microliters of this suspension was distributed in four tubes, Cefinase and Cefinase Plus disks were added, and the

TABLE 1. β-Lactamase testing of *B. wadsworthia* strains comparing three different media, Cefinase and Cefinase Plus disks, and aerobic and anaerobic testing conditions

Incubation time	Test medium	Tube assay[a] with:								Disk assay[b] with aerobic incubation			
		Anaerobic incubation				Aerobic incubation							
		Cefinase		Cefinase Plus		Cefinase		Cefinase Plus		Cefinase		Cefinase Plus	
		No. positive (n = 14)[c]	Avg intensity	No. positive (n = 14)	Avg intensity	No. positive (n = 14)	Avg intensity	No. positive (n = 14)	Avg intensity	No. positive (n = 14)	Avg intensity	No. positive (n = 14)	Avg intensity
15 min	Brucella-pyruvate	11	3.1	13	3.5	10	2	13	2.1	13	1.9	13	1.9
	Brucella-taurine	6	0.58	8	0.77	1	0.04	1	0.04	0	0	0	0
	Brucella	11	0.7	11	0.70	4	0.23	5	0.38	12	1.0	13	1.0
30 min	Brucella-pyruvate	13	3.7	13	3.9	13	2.4	13	2.6	13	2.0	13	2.0
	Brucella-taurine	7	1.0	9	1.4	1	0.04	3	0.23	1	0.08	0	0
	Brucella	11	1.2	13	1.3	10	0.58	13	0.85	13	1.6	13	1.6
1 h	Brucella-pyruvate	13	3.7	13	4.5	13	3.7	13	3.9	13	2.0	13	2.0
	Brucella-taurine	8	1.5	11	2.1	1	0.23	5	0.46	5	0.54	9	0.69
	Brucella	13	1.6	13	1.8	13	1.1	13	1.4	13	1.6	13	1.6
2 h	Brucella-pyruvate	12	2.9	13	3.7	13	3.9	13	4.5	13	2.0	13	2.0
	Brucella-taurine	10	2.4	12	2.8	3	0.35	5	0.69	6	0.62	10	0.77
	Brucella	13	1.9	13	2.1	13	1.1	13	1.4	13	1.6	13	1.7
3 h	Brucella-pyruvate	10	1.8	10	2.2	13	3.7	13	4.0	ND[d]	ND	ND	ND
	Brucella-taurine	10	1.9	11	2.1	4	0.42	5	0.65	ND	ND	ND	ND
	Brucella	13	2.1	13	2.3	13	1.5	13	1.8	ND	ND	ND	ND

[a] Color development was visually graded on the basis of the intensity of red color produced, from 0 (no color change) to 5 (strong red color).
[b] Color development was visually graded from 0 to 2: 0, no color change; 1, pink color; 2, strong red color.
[c] Includes β-lactamase-negative strain (WAL 7813) and 13 β-lactamase-positive strains (WAL 7959, WAL 8114, WAL 8126, WAL 8144, WAL 8182, WAL 8280, WAL 8448, WAL 8969, WAL 9046, WAL 9077, WAL 9118, WAL 9159, WAL 9176).
[d] ND, not done.

tubes were vortexed. One set of the tubes was incubated inside an anaerobic chamber, and one set was incubated aerobically at +35°C. The color change was inspected visually for 3 h (Table 1) and scored from 0 to 5 on the basis of intensity.

Cefinase and Cefinase Plus disk assays. β-Lactamase production was measured for strains grown for 48 h on the three brucella agar media by the chromogenic cephalosporin disk method (Cefinase and Cefinase Plus; BBL), as described by the manufacturer. A loopful of bacterial mass from brucella-pyruvate and -taurine plates was inoculated on the disks placed in empty petri dishes, followed by a drop of distilled water. *B. wadsworthia* grows very poorly on brucella agar without pyruvate or taurine. Therefore, several plates were inoculated, a heavy suspension (with a McFarland standard of >7) of the growth was harvested in distilled water, and 50 μl of this suspension was inoculated onto the disks. The test disks were incubated at room temperature aerobically and anaerobically, with the petri dish lids closed to prevent drying. The reaction was monitored for up to 2 h and visually scored.

Penicillin (2-U) disk assays. The strains were grown for 48 h on brucella and brucella-pyruvate. From the growth on each medium, a suspension equal to McFarland standard 0.5 was prepared in 3-ml dilution blank tubes (Anaerobe Systems, San Jose, Calif.). By using the Kirby-Bauer technique, brucella and brucella-pyruvate plates were inoculated, and penicillin disks were placed on the plates. The plates were incubated anaerobically for 72 h. The zones around the disks were measured, and a zone diameter of >20 mm was considered to indicate sensitivity (6).

Susceptibility studies. The antimicrobial susceptibility studies were performed by using the NCCLS-approved Wadsworth agar dilution technique outlined in NCCLS document M11-A4 (8) and the spiral gradient technique (User guide, Spiral System Instruments, Bethesda, Md.). *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741 were included for quality control. All of the MIC determinations were performed on brucella agar supplemented with 1% pyruvic acid to enhance the growth of *B. wadsworthia*. The MICs were interpreted with the aid of triphenyltetrazolium chloride as described previously (13). The precise penicillin G MICs for strains WAL 7966 and WAL 9009 were also tested with the Etest method (3).

RESULTS

Cefinase and Cefinase Plus quantitative assays. The results for Cefinase and Cefinase Plus tube tests are listed in Table 1. The β-lactamase-negative strain included as an internal quality control (WAL 7813) remained negative in all the tests. The color development (intensity) on tests from brucella-pyruvate plates was faster than that from brucella or brucella-taurine plates. Anaerobic incubation generally yielded faster and stronger reactions. The reactions were always equal to or somewhat stronger with the Cefinase Plus method than with the Cefinase method.

Cefinase and Cefinase Plus disk assays. Table 1 lists the results for conventional Cefinase disk assays. There was no significant difference between the anaerobic and aerobic incubations with the disk assays; therefore, only the aerobic results are shown here. The average intensities of tests from brucella-pyruvate plates were stronger than those from brucella or brucella-taurine plates. All β-lactamase-producing strains from brucella-pyruvate and brucella plates, but only one from brucella-taurine plates, turned positive after 30 min of incubation.

Penicillin (2-U) disk assays. The zones around penicillin 2 unit disks obtained on brucella and brucella-pyruvate plates are listed in Table 2. Overall, the zone diameters were a few millimeters larger on brucella-pyruvate plates. Three β-lactamase-negative strains (WAL 7813, WAL 8658, and WAL 9344) showed zone diameters of 27 mm on brucella-pyruvate plates and of 27, 25, and 16 mm on brucella plates, respectively. All but one of the β-lactamase-producing strains produced zones of ≤20 mm, and β-lactamase-negative strains produced zones of >20 mm. The one exception was the β-lactamase-positive strain WAL 7966; it showed a 22-mm-diameter zone around the penicillin (2-U) disk on brucella-pyruvate (18 mm on brucella), and the penicillin MIC for this strain was 0.5 μg/ml. The zones on brucella plates were difficult to interpret due to a very

TABLE 2. Correlation of *B. wadsworthia* β-lactamase, penicillin (2-U) disk zone diameters, and penicillin G and ampicillin MICs

Isolate no. or group	β-Lactamase production	Penicillin (2-U) disk (zone diam or range [mm])		MIC or range (μg/ml)		
		Brucella	Brucella-pyruvate	Penicillin G	Ampicillin	Ampicillin-sulbactam[a]
WAL 7813	−	27	27	0.25	0.5	0.25/0.125
WAL 8658	−	25	27	0.25	0.5	0.25/0.125
WAL 9344	−	16	27	0.25	0.5	25/0.125
WAL 7966	+	18	22	0.5	4	2/1
WAL 9009	+	17	20	0.5	4	2/1
WAL 11162	+	15	15	2	8	2/1
WAL 11245	+	15	16	2	8	2/1
Four strains[b]	+	0	14–19	1–4	4–32	0.5/0.25–2/1
Thirty strains[c]	+	0	0	2–32	2–>64	0.5/.25–4/2

[a] The ampicillin/sulbactam ratio was 2:1.
[b] Strains WAL 8448, WAL 9046, WAL 10695, and WAL 11649.
[c] Strains WAL 7959, WAL 8114, WAL 8136, WAL 8144, WAL 8283, WAL 8380, WAL 8551, WAL 8697, WAL 8712, WAL 8839, WAL 8935, WAL 8961, WAL 8969, WAL 9077, WAL 9128, WAL 9159, WAL 9176, WAL 9294, WAL 9693, WAL 9798, WAL 9965, WAL 9974, WAL 10385, WAL 10597, WAL 10876, WAL 10918, WAL 11395, WAL 11532, WAL 11615, and WAL 11640.

slight, transparent growth pattern. On brucella-pyruvate plates, the growth was heavier and the zones were easy to read.

Susceptibility studies. The results of the antimicrobial susceptibility studies are listed in Table 2. Thirty-six strains were resistant to penicillin, and thirty-eight were resistant to ampicillin (MIC > 0.5). The addition of sulbactam lowered the ampicillin MICs two- to sixfold. The MICs of penicillin for five strains, including two β-lactamase-positive strains (WAL 7966 and WAL 9009), indicated susceptibility (MIC ≤ 0.5). Penicillin G MICs for both WAL 7966 and WAL 9009 were 0.38 μg/ml each as determined by the Etest.

DISCUSSION

Our previous study showed that 87% of *B. wadsworthia* strains were β-lactamase positive (13). Similarly, a recent report found 90.8% of 87 *B. wadsworthia* strains to be β-lactamase positive (12). The present study suggests that the incidence could be even higher: of 21 strains deposited in the culture collection as β-lactamase negative, only three remained negative after repeated testing when prolonged incubation and a heavy inoculum from brucella-pyruvate medium were used. These strains were within the first 120 *B. wadsworthia* strains deposited in the Wadsworth Anaerobe Laboratory collection, thus suggesting that the incidence of β-lactamase production may actually exceed 97%.

B. wadsworthia β-lactamase is a cephalosporinase and is nonreactive with the acidometric penicillinase test (Summanen et al., unpublished data). The chromogenic cephalosporin nitrocefin has been found to be effective in detecting all known β-lactamases (10). The Cefinase and Cefinase Plus methods use chromogenic cephalosporin nitrocefin and a nonnitrocefin chromogenic cephalosporin, respectively. *B. wadsworthia* β-lactamase was detected with 100% agreement by the Cefinase and Cefinase Plus methods; the Cefinase Plus method generally produced a color intensity equal to or stronger than that of the Cefinase method. Similar results have been reported in previous evaluations of various other anaerobic and aerobic organisms (4, 7).

B. wadsworthia grows luxuriantly on taurine-containing media (Laue et al., Abstr. Anaerobe Soc. Am. Congr. Anaerobic Bacteria 1998, p. 11), and an adequate inoculum for β-lactamase tests was easy to obtain from brucella-taurine plates. However, the detection of β-lactamase was clearly affected by taurine, since 62% of the strains remained negative in the aerobic tube tests. Anaerobic testing conditions markedly enhanced the detection of β-lactamase production from strains on this medium, but three strains still remained negative at 3 h. The demonstration of β-lactamase production with the conventional disk test from brucella-taurine plates was slow and insensitive, and the reactions were difficult to interpret since the colonies on this medium were dark grey, which interfered with interpretation of the test.

The growth pattern of *B. wadsworthia* is very fastidious on commonly used nonselective media, such as brucella and CDC agars. For demonstration of β-lactamase production from brucella medium, several plates were required to obtain adequate inoculum. The growth of *B. wadsworthia* is enhanced on brucella-pyruvate plates, and brucella-pyruvate is the recommended medium for susceptibility testing (13). The β-lactamase tests done with this medium produced stronger and faster reactions than those done with brucella or brucella-taurine. The tube test was clear and easy to interpret, and the color intensities were significantly higher at 15 min to 1 h than those with the other test media. Anaerobic incubation led to faster reactions, but after 1 h of incubation, the color intensity in some of the test tubes started fading; this did not occur in tubes incubated aerobically until after 3 h of incubation. The conventional disk test produced positive reactions within 15 min. As with the brucella-taurine medium, but to a lesser degree, the greyish colony color sometimes interfered with reading the disk tests when brucella-pyruvate plates were used.

The penicillin and ampicillin MICs obtained correlated with β-lactamase production and the NCCLS guidelines (8), with the exception of those for two strains for which β-lactamase production was demonstrated but for which the penicillin MICs were in the susceptible range (0.5 μg/ml). However, ampicillin MICs were 4 μg/ml for these strains, indicating resistance. According to the NCCLS guidelines, β-lactamase-positive organisms should be considered resistant to penicillin regardless of the MIC (8). Sulbactam significantly lowered the ampicillin MICs, further demonstrating that *B. wadsworthia* β-lactam resistance is due to β-lactamase.

Penicillin (2-U) disk susceptibilities correlated with β-lactamase production and MICs: a strain producing a zone of ≤20 mm should be suspected to be β-lactamase positive and confirmed by β-lactamase testing. The penicillin (2-U) disk is generally used for susceptibility testing of aerobic bacteria, but

it has also been reported to be useful in screening for β-lactamase production in anaerobic gram-negative bacilli (6, 9).

In conclusion, the results of this study indicate that a vast majority (97%) of *B. wadsworthia* strains produce β-lactamase and that routine β-lactamase testing in the clinical laboratory may not even be necessary. The β-lactamase production by *B. wadsworthia* was most reliably demonstrated with pyruvate-containing medium. Brucella supplemented with taurine was unsuitable for *B. wadsworthia* β-lactamase detection. The tube test was easier to interpret than the conventional disk test. The reaction occurred faster anaerobically; however, aerobic incubation yielded comparable results. *B. wadsworthia* β-lactamase tests should be interpreted after 30 min of incubation. In detecting β-lactamase, Cefinase and Cefinase Plus tests were in agreement; Cefinase Plus yielded somewhat faster reactions.

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